P2Y₁₂ polymorphisms and antiplatelet effects of aspirin in patients with coronary artery disease

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WHAT IS ALREADY KNOWN ABOUT THIS SUBJECT

- Genetic polymorphisms of the *P2Y₁₂* ADP receptor on platelets have been shown to contribute to variability in platelet aggregation in healthy humans.
- *P2Y₁₂* ADP receptor polymorphisms are more frequently present in patients with vascular disease than in healthy people.
- The majority of patients with vascular disease receive acetylsalicylic acid as an anti-aggregatory agent, which has also been shown to induce a variable response; however, the role of *P2Y₁₂* ADP receptor polymorphisms in the platelet response to acetylsalicylic acid in patients with vascular disease has not yet been studied.

WHAT THIS STUDY ADDS

- The present data show that the platelet response to acetylsalicylic acid is independent of the presence or absence of *P2Y₁₂* ADP receptor polymorphisms in patients with stable coronary artery disease who have had their first myocardial infarction.
- This is important, as studies in healthy humans had suggested that carriers of *P2Y₁₂* ADP receptor polymorphisms may be at increased risk of experiencing cardiovascular events.
- However, the observed variability of the platelet response to the cyclooxygenase inhibitor acetylsalicylic acid (in our study) and to the *P2Y₁₂* ADP receptor blocker clopidogrel (in a study by Angiolillo *et al.* [18]) in patients with coronary artery disease is clearly not determined by common *P2Y₁₂* ADP receptor polymorphisms.

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Keywords

ADP receptors, aspirin, coronary artery disease, pharmacogenetics, platelet aggregation

Received

25 January 2007

Accepted

19 August 2007

Published OnlineEarly

8 November 2007

AIMS

Recently, two genetic polymorphisms of the platelet ADP receptor *P2Y₁₂* (haplotypes H2 and 34T) have been implicated in increased platelet aggregation and atherothrombotic risk. It was suggested that these polymorphisms contribute to a diminished response to antiplatelet drugs. Therefore, we investigated the effects of these polymorphisms on platelet aggregation in aspirin-treated patients with coronary artery disease (CAD).

METHODS

Platelet aggregation was studied in platelet-rich plasma from 124 patients with CAD treated with 100 mg aspirin day⁻¹. *P2Y₁₂* ADP receptor polymorphisms were determined by PCR-RFLP. The 52G > T polymorphism was used as tag-SNP for the H2 haplotype. Aggregation was induced by 1 mg l⁻¹ collagen. In a subgroup (*n* = 72), a concentration-response curve to collagen (0.5–10 mg l⁻¹), aggregation at 2 µmol l⁻¹ ADP and 1 mmol l⁻¹ arachidonic acid were determined.

RESULTS

Whereas arachidonic acid-induced aggregation was inhibited in all patients, collagen and ADP-induced aggregation were highly variable. However, aggregation did not differ significantly between carriers and noncarriers of the 52T-allele (1 mg l⁻¹ collagen: 32.7% (21.9–38.6%) vs. 32.5% (21.2–41.6%); *P* = 0.77; ADP: 33.1% (29.9–40.9%) vs. 39.1% (31.5–49.7%); *P* = 0.34), respectively. EC₅₀ values were 1.26 mg l⁻¹ (0.79–2.02) and 1.54 mg l⁻¹ (0.98–2.4) collagen in noncarriers and carriers of the H2 haplotype, respectively (*P* = 0.56). Moreover, the 34°C > T polymorphism did not significantly affect any of the aggregatory responses.

CONCLUSIONS

Low-dose aspirin inhibits platelet aggregation to the same extent in patients carrying or not carrying the *P2Y₁₂* H2 haplotype and/or the 34T allele. Our data do not support the hypothesis that these polymorphisms contribute to an attenuated antiplatelet effect of aspirin.

Introduction

Antiplatelet drugs are a mainstay in the treatment of cardiovascular diseases. However, response to antiplatelet drugs is highly variable and this variability may affect clinical outcome [1, 2]. One of the key molecules in platelet aggregation is adenosine diphosphate (ADP), which enhances platelet aggregation and is released from activated platelets. There are three known ADP receptors on platelets: the $P2X_1$ receptor (a ligand-induced ion channel) and the G-protein coupled receptors $P2Y_1$ and $P2Y_{12}$ [3]. The $P2Y_{12}$ ADP receptor has been cloned recently. It is the target of the thienopyridine antiplatelet drugs clopidogrel and ticlopidine [4]. Stimulation of this receptor facilitates fibrinogen binding to the glycoprotein (GP) IIb-IIIa receptor, leading to reversible primary platelet aggregation, and finally to irreversible secondary aggregation (Figure 1) [5].

The influence of genetic factors in general and platelet receptors in particular on platelet aggregability is only partially understood at present [6]. Genetic polymorphisms of the $P2Y_{12}$ ADP receptor have been described which may influence the activation of this receptor by ADP or the response of patients to platelet inhibitors. Whereas rare mutations within the $P2Y_{12}$ gene cause a bleeding diathesis [7, 8], common polymorphisms of the $P2Y_{12}$ gene may have important implications in atherothrombosis [9–11].

The $P2Y_{12}$ gene spans 47 kb and comprises three exons [12]. At present, nine common polymorphisms in the $P2Y_{12}$

gene have been described, five of which are in complete linkage disequilibrium. The rarer variant is denoted H2 haplotype, whereas the wildtype haplotype is named H1 [9, 13]. Another frequent polymorphism which was found to be associated with a reduced clinical response to thienopyridines is the $34^{\circ}\text{C} > \text{T}$ polymorphism [11].

In a recent study by Fontana *et al.* [9], the H2 haplotype of the $P2Y_{12}$ ADP receptor was significantly associated with increased ADP-induced platelet aggregation in healthy volunteers. The study demonstrated increased platelet aggregability in homo- and heterozygous carriers of the H2 allele in a gene-dose dependent manner. Moreover, in a case-control study, the H2 haplotype was over-represented in 184 patients with peripheral arterial disease as compared with 330 age-matched controls [10]. These investigations have suggested that carriers of the H2 haplotype may be prone to an increased risk of atherothrombosis and to a diminished response to antiplatelet drugs. Moreover, since thienopyridines like clopidogrel provide only partial $P2Y_{12}$ blockade [14, 15], patients with increased $P2Y_{12}$ activity may benefit less from treatment with these compounds. Thus, it has been suggested that $P2Y_{12}$ polymorphisms are associated with less protection by these platelet inhibitors [9], which would be of considerable clinical relevance for patients with coronary artery disease (CAD). However, the majority of patients with CAD receive treatment with acetylsalicylic acid, which primarily inhibits the thromboxane (TXA_2)-dependent signalling cascade of platelet activation. For acetylsalicylic acid, variable responses of platelet inhibition were reported like for clopidogrel [16]. As there is crosstalk between ADP- and TXA_2 -dependent pathways of platelet activation (i.e. stimulation of thromboxane receptors on platelets leads to ADP release and vice versa, Figure 1), the role of $P2Y_{12}$ ADP receptor polymorphisms in the response to acetylsalicylic acid is unclear.

Do mutations in the $P2Y_{12}$ gene for the ADP receptor result in general anergy of platelets, or do they affect specific pathways of platelet activation? Study of $P2Y_{12}$ gene mutations may allow investigation of crosstalk between signalling of different activation pathways such as ADP and arachidonic acid. We hypothesized that the anti-aggregatory effects of aspirin may be attenuated in patients carrying $P2Y_{12}$ ADP receptor polymorphisms due to predominance of ADP-dependent aggregatory mechanisms (Figure 1). We therefore investigated whether frequent genetic $P2Y_{12}$ polymorphisms are associated with increased *ex vivo* platelet aggregation during long-term aspirin treatment in patients with CAD.

Methods

Study population

A total of 140 patients were initially investigated. Inclusion criteria were stable CAD (documented by coronary

Figure 1

Study hypothesis. Platelet aggregation is mediated by multiple pathways. The two most important pathways are the cyclooxygenase (COX)-dependent formation of thromboxane A_2 (TxA_2), which binds to thromboxane receptors, and the granule secretion of ADP, which binds to ADP receptors (e.g. $P2Y_{12}$). Both pathways lead to GPIIb-IIIa receptor activation resulting in stable aggregate formation. During aspirin treatment, the TxA_2 mediated pathway is blocked, whereas the ADP mediated pathway is largely unaffected. In the presence of $P2Y_{12}$ receptor polymorphisms (H2 haplotype and/or $34^{\circ}\text{C} > \text{T}$), which were recently associated with increased platelet aggregation, aspirin treatment may be ineffective due to predominance of the ADP mediated pathway

angiography and/or previous myocardial infarction) and continuous use of 100 mg aspirin day⁻¹. Patients with myocardial infarction within the last 2 weeks, patients using other anti-aggregatory drugs (e.g. clopidogrel) and non-compliant patients (see below) were excluded. Other exclusion criteria were surgery within the last 3 months, renal failure, liver insufficiency, and alcohol or drug abuse. One hundred and twenty-four patients constituted the final study cohort. Twelve patients were excluded due to the presence of one or more of the clinical exclusion criteria, and four patients were excluded due to a significant remaining effect of *in vitro* added aspirin (see below). The study was approved by the local Ethics Committee, and all patients gave written informed consent.

Genotyping

Genomic DNA was isolated from whole blood by using the QIAamp DNA Blood Mini Kit (Qiagen, Germany) according to the manufacturer's instructions. A forced-mutation polymerase chain reaction (PCR)-based restriction fragment length polymorphism (RFLP) analysis was developed to detect the *P2Y₁₂* gene polymorphisms. The 52G > T polymorphism was used as tag-SNP for the H2 haplotype. The primers sets used were: 5'aataataatTCACCTCTGCGCCCGG3'/5'CCGG ATTTGAAAGAAAATCCTCA3' for the 52G > T polymorphism, and 5'TTTAGAGGAGGCTGTGTCCAA3'/5'aataatGTTACCAGGCGCAGAGGTGAA3' for the 34°C > T polymorphism, respectively. Forced mutations are underlined and were introduced to yield allele-specific restriction patterns. PCR was performed with 1.25 U Sawady-Taq polymerase in Buffer Y (both Peqlab, Germany) with final concentrations of 250 µM dNTP and 1 µM of primers in a reaction volume of 25 µl. A Perkin-Elmer 9600 (Perkin-Elmer, Germany) thermal cycler was used. The thermal cycling conditions comprised an initial denaturation step at 94°C for 3 min and 35 cycles at 94°C for 20 s, 58°C for 20 s, and 72°C for 25 s. Final extension was performed at 72°C for 3 min. The amplification product for the detection of the 52G > T polymorphism was subjected to Sma I-digestion (New England Biolabs, Germany). The PCR-product used to detect the 34°C > T polymorphism was digested with Tsp509 I (New England Biolabs, Germany). The products were size-fractionated on a 3.0% small-DNA agarose gel (Biozym, Germany). Sizes of the PCR-products after enzymatic digestion are given in Table 1. All 124

patients were successfully genotyped for both polymorphisms.

Determination of platelet aggregation

Citrated venous blood samples were collected after an overnight fast. Platelet-rich plasma (PRP) was prepared by centrifugation at 200 g for 15 min at ambient temperature. Platelet-poor plasma (PPP) was prepared from the remaining volume of blood by centrifugation at 2000 g for 10 min. Aliquots (250 µl) of PRP were incubated for 15 min at 37°C before adding collagen (Nycomed, Germany), ADP (Serva, Germany), or arachidonic acid (Rolf Greiner Bio-Chemica, Germany). Platelet aggregation was monitored for 4 min at 37°C as an increase in light transmission using an Apact aggregometer (LABOR, Germany) [17]. All measurements were run in duplicate for each stimulus/inhibitor combination. Final agonist concentration in the reactions were: 1 mg l⁻¹ collagen (in all 124 patients), 0.5, 1, 2, 5 and 10 mg l⁻¹ collagen (in 75 patients), and 2 µmol l⁻¹ ADP and 1 mmol l⁻¹ arachidonic acid (in 72 patients). As primary variables, we analyzed the amplitude and the slope of the time-response curves. Both values for the amplitude and the slope yielded the same results. Data are only shown for the amplitude.

To detect patients with remaining aspirin-sensitive aggregation (e.g. due to noncompliance or pharmacokinetic reasons) PRP was challenged *in vitro* with aspirin (final concentration 100 µmol l⁻¹; Sigma, Germany) for 15 min. Subsequently, aggregation was determined in the presence of 1 mg l⁻¹ collagen as agonist. In four patients, aggregation was clearly reduced after pre-incubation with aspirin. These patients were excluded from the presented analyses.

Statistical analysis

Differences between genotype groups were analyzed by using the nonparametric Mann-Whitney *U*-test. Data are depicted as box plots giving 10th, 25th, 50th (median), 75th and 90th percentile for each genotype. Sigmoidal dose-response curves were calculated and compared with Prism 4 (GraphPadSoftware Inc., USA). StatView 5.0 (SAS Institute) was used for other statistical analyses. *P* < 0.05 was considered statistically significant.

Results

Pertinent clinical characteristics of the study population are shown in Table 2. Allele frequencies for the 52G > T and the 34°C > T polymorphism of the *P2Y₁₂* ADP receptor gene were 0.84 for the 52G-allele, and 0.70 for the 34°C-allele, respectively. Genotype distributions followed Hardy-Weinberg's law. Genotype distributions of the study population are given in Table 3. Because of the low number of patients homozygous for the 52T-allele (*n* = 2) and 34T-allele (*n* = 11), and because aggregation data for homozy-

Table 1

Fragment sizes of PCR-products after enzymatic digestion

Polymorphism	W	R
52G > T	129 + 23	152
34°C > T	156	126 + 30

W denotes the more frequent 'wild-type' allele; R denotes the rarer allele. Values indicate length in base pairs.

Table 2Pertinent patient characteristics in the study group ($n = 124$)

Parameter	
Age (years)	66.1 ± 8.1
Male, n (%)	104 (83.9)
Body mass index (kg m^{-2})	26.6 ± 3.7
Hypercholesterolemia, n (%)	106 (85.5)
Hypertension, n (%)	109 (87.8)
Diabetes, n (%)	15 (12.1)
Current smokers, n (%)	5 (4.0)
History of MI, n (%)	86 (69.4)
LDL cholesterol (mg dl^{-1})	102.1 ± 30.5
HDL cholesterol (mg dl^{-1})	53.7 ± 13.9
Triglycerides (mg dl^{-1})	116.6 ± 64.4
C-reactive protein (mg l^{-1})	1.2 (0.7–2.7)
Systolic blood pressure (mm Hg)	139.9 ± 19.0
Diastolic blood pressure (mm Hg)	83.0 ± 10.3

Continuous variables are given as mean $\pm$ SD except for C-reactive protein which is given as median and interquartile range.

Table 3Frequencies of $P2Y_{12}$ receptor genotypes in 124 patients with coronary artery disease in this study group

Polymorphism	Genotype	Frequency, n (%)
52G > T	GG	86 (69.4)
	GT	36 (29.0)
	TT	2 (1.6)
34°C > T	CC	60 (48.4)
	CT	53 (42.7)
	TT	11 (8.9)

gous patients (52TT, 21.6–30.7%; 34TT, 21.3–33.7%) fell within the range of heterozygous patients (52GT, 22.0–40.3%; 34CT, 22.4–40.5%), data were pooled with those obtained in the heterozygous patients for the analysis of aggregation data.

A wide interindividual variability of maximal *ex vivo* platelet aggregation responses to collagen and ADP in PRP from aspirin-treated patients was noted (Figure 2). Individual collagen- and ADP-induced platelet aggregation were significantly correlated ($r = 0.63$, $P < 0.0001$), as was collagen- and arachidonic acid-induced aggregation ($r = 0.73$, $P < 0.0001$). In contrast, arachidonic acid-induced aggregation was inhibited in all patients (Figure 2). This indicated that cyclo-oxygenase pathways were inhibited by aspirin in these patients.

Maximal aggregation in response to 1 mg l^{-1} collagen was not different between the 38 patients carrying one or two 52T-alleles and the 86 patients carrying no 52T-allele, with median maximal aggregation of 32.7% (21.9–38.6%) vs. 32.5% (21.2–41.6%, $P = 0.77$; Figure 3A). We found a similar result for the 34°C > T polymorphism, with a median

Figure 2

Interindividual variability of maximal platelet aggregation in aspirin-treated patients with coronary artery disease (CAD). Maximal aggregation (%) in response to 1 mg l^{-1} collagen (x-axis) was plotted against maximal aggregation (%) in response to $2 \mu\text{mol l}^{-1}$ ADP (●), and 1 mmol l^{-1} arachidonic acid (○), respectively, in 72 CAD patients treated with 100 mg aspirin day^{-1} . Collagen- and ADP-induced aggregation were significantly correlated ($r = 0.63$; $P < 0.0001$). Similarly, collagen- and arachidonic acid-induced aggregation were correlated ($r = 0.73$; $P < 0.0001$). However, aggregation induced by arachidonic acid was considered to be inhibited, because none of the patients had an aggregation response above 15% and subjects without aspirin therapy showed significantly higher maximal aggregation values (data not shown).

maximal aggregation of 33.4% (22.3–38.9%) for 64 carriers of the 34T-allele vs. 31.4% (21.2–42.1%) for 60 noncarriers of the 34T-allele ($P = 0.94$; Figure 3B).

In a subgroup of 75 patients, a concentration-response curve for collagen-induced aggregation was carried out. The response curves of 17 carriers in comparison with 58 noncarriers of the 52T-allele were virtually superimposed (Figure 4A). EC_{50} values were 1.26 mg l^{-1} collagen (0.79–2.02) and 1.54 mg l^{-1} (0.98–2.4) in noncarriers and carriers of the H2 haplotype, respectively ($P = 0.56$). Similarly, we observed no significant difference between the concentration response curves of 42 carriers and 33 noncarriers of the 34T-allele (Figure 4B). The EC_{50} values for collagen were 1.6 mg l^{-1} (1.2–2.17 95% CI) and 1.2 mg l^{-1} (0.7–1.9) in noncarriers and carriers of the 34T-allele, respectively ($P = 0.25$).

The influence of $P2Y_{12}$ polymorphisms on platelet aggregation induced by ADP was assessed at an ADP concentration of $2 \mu\text{mol l}^{-1}$. In previous studies in healthy untreated volunteers, the strongest association between the $P2Y_{12}$ H2 haplotype and maximal aggregation was found at this concentration [9]. In our aspirin-treated study population, we did not detect any significant differences in aggregation response to ADP in patients carrying the

Figure 3

Maximal collagen-induced platelet aggregation in platelets with $P2Y_{12}$ genotypes in aspirin-treated patients with coronary artery disease (CAD). Maximal aggregation (%) in response to 1 mg l^{-1} collagen in CAD patients treated with $100 \text{ mg aspirin day}^{-1}$. (A) patients carrying no 52T-allele ($n = 86$) or one or two 52T-alleles ($n = 38$). (B) patients carrying no 34T-allele ($n = 60$) or one or two 34T-alleles ($n = 64$)

mutated 52T-allele ($n = 16$) in comparison with patients carrying no 52T-allele ($n = 56$). Median values of maximal aggregation were 33.1% (29.9–40.9%) for carriers and 39.1% (31.5–49.7%) for noncarriers of the 52T-allele ($P = 0.34$; Figure 5A). Similarly, we observed no significant association between the $34^{\circ}\text{C} > \text{T}$ polymorphism and ADP-induced aggregation in 39 patients carrying and 33 patients carrying no 34T-allele, with median maximal aggregation of 39.1% (30.8–50.7%) vs. 34.2% (30.4–43.6%) ($P = 0.24$; Figure 5B).

Of the 124 investigated patients 10.5% (13 patients) were carriers of both polymorphisms (52T and 34T). Between these patients carrying both polymorphisms and

Figure 4

Concentration-response curves for collagen-induced platelet aggregation subject to $P2Y_{12}$ genotypes in aspirin-treated patients with coronary artery disease (CAD). Maximal aggregation (%) in response to 0.5, 1, 2, 5 and 10 mg l^{-1} collagen in CAD patients treated with $100 \text{ mg aspirin day}^{-1}$. (A) patients carrying no 52T-allele ($n = 58$) or carrying one or two 52T-alleles ($n = 17$; $P = 0.56$) (52 G/G, Δ ; 52 G/T or 52 T/T, $\circ$). (B) patients carrying no 34T-allele ($n = 33$) or one or two 34T-alleles ($n = 42$; $P = 0.25$). Data are mean $\pm$ SEM (34 C/C, Δ ; 34 C/T or 34 T/T, $\circ$)

patients carrying none of the investigated polymorphisms (28.2% of the study population; $n = 35$), we also observed no significant differences in aggregation response to 1 mg l^{-1} collagen, with median maximal aggregation of 33.1% (22.4–39.6%) vs. 27.4% (20.7–42.8%) ($P = 0.84$; Figure 6). Similar results were obtained at the other collagen concentrations and $2 \mu\text{mol l}^{-1}$ ADP, respectively (data not shown).

Figure 5

Maximal ADP-induced platelet aggregation subject to $P2Y_{12}$ genotypes in aspirin-treated patients with coronary artery disease (CAD). Maximal aggregation (%) in response to $2 \mu\text{mol l}^{-1}$ ADP in CAD patients treated with $100 \text{ mg aspirin day}^{-1}$. (A) patients carrying no 52T-allele ($n = 56$) or one or two 52T-alleles ($n = 16$). (B) patients carrying no 34T-allele ($n = 33$) or one or two 34T-alleles ($n = 39$)

Discussion

In our study, *ex vivo* platelet aggregation of CAD patients continuously treated with aspirin (100 mg day^{-1}) was not significantly affected by the $52\text{G} > \text{T}$ polymorphism of the ADP receptor gene $P2Y_{12}$ (H2 haplotype). These findings are in apparent contrast to a recent study by Fontana *et al.* [9] who found enhanced aggregation response to ADP in healthy untreated volunteers who were carriers of the H2 haplotype. These authors suggested that this might put patients at increased risk of thrombotic complications despite ongoing antiplatelet therapy. Contrary to these expectations, in our study carriers of the H2 haplotype had no significantly diminished response to aspirin as com-

Figure 6

Maximal collagen-induced platelet aggregation in aspirin-treated patients with coronary artery disease (CAD) carrying both polymorphisms, 52T and 34T. Maximal aggregation (%) in response to 1 mg l^{-1} collagen in CAD patients treated with $100 \text{ mg aspirin day}^{-1}$ carrying neither the $52\text{G} > \text{T}$ polymorphism nor the $34\text{C} > \text{T}$ polymorphism ($n = 35$), and aspirin-treated patients carrying both polymorphisms, $52\text{G} > \text{T}$ and $34\text{C} > \text{T}$ ($n = 13$)

pared with noncarriers. This was true for both, collagen- and ADP-induced platelet aggregation, which were correlated closely. This finding is in accordance with the findings of Angiolillo and coworkers who recently determined platelet aggregation response to collagen and ADP in 119 CAD patients on clopidogrel [18]. They found no differences between carriers and noncarriers of the $744\text{T} > \text{C}$ polymorphism, which is also part of the H2 haplotype, for all of the assessed platelet function assays.

The second polymorphism in the $P2Y_{12}$ gene investigated ($34\text{C} > \text{T}$) was also not associated with the maximal aggregation response in our aspirin-treated study population. This was in line with the findings of Fontana *et al.* and also with a clinical study [11] in which neither the $34\text{C} > \text{T}$ nor the $52\text{G} > \text{T}$ polymorphism were associated with a higher risk for ischaemic cerebrovascular events in patients with peripheral arterial disease on aspirin treatment. Surprisingly, in the same study, in patients during continuous treatment with clopidogrel, carriers of the 34T-allele had a four-fold increased risk for neurological events compared with noncarriers.

The discrepancy between our results and those of Fontana *et al.* [9] concerning the aggregation response in carriers of the H2 haplotype may be explained as follows: Whereas Fontana *et al.* [9] investigated subjects without antiplatelet therapy, our patients were treated with aspirin. Recently, it has been shown that stimulation of the $P2Y_{12}$ ADP receptor leads to an enhanced thromboxane production [19–21]. This mechanism is absent in our aspirin-treated patients due to complete inhibition of COX-1 in platelets, as evidenced by the lack of platelet response to

arachidonic acid. Thus, the results of Fontana *et al.* [9] and our findings may imply that the H2 haplotype is particularly involved in an enhancement of thromboxane generation. This would lead to higher maximal aggregation in untreated subjects, whereas aggregation is not affected in patients on aspirin therapy. Further studies should be undertaken using selective thromboxane receptor antagonists to investigate this pathway. In another study, Hetherington *et al.* [13] investigated the H2 haplotype and a further three of the common P2Y₁₂ polymorphisms by a flow cytometric fibrinogen binding assay in volunteers without antiplatelet therapy. In this assay, none of the polymorphisms was found to affect fibrinogen binding to activated GP IIb-IIIa receptors. However, fibrinogen binding is more directly linked to ADP receptor activation and is unaffected by thromboxane generation. Thus, the results of these studies and our study are in line, if the H1/H2 polymorphism is of particular importance for thromboxane formation after P2Y₁₂ stimulation.

P2Y₁₂ ADP receptor polymorphisms are more frequent in patients with vascular disease, the majority of whom receive aspirin. In this study, we report that chronic aspirin treatment provides the same degree of inhibition of platelet aggregation in CAD patients carrying and not carrying the H2 haplotype and the 34°C > T polymorphism of the P2Y₁₂ ADP receptor gene, respectively. This has implications relating to the safety and efficacy of aspirin and antiplatelet therapy: in line with the data from Ziegler *et al.* [11], which assessed clinical end-points, aspirin treatment is also of benefit for secondary prevention in carriers of the investigated P2Y₁₂ gene polymorphisms. Our study suggests that the investigated polymorphisms in P2Y₁₂ do not contribute to aspirin nonresponsiveness to a major extent. This study confirms the existence of separate and independent platelet signalling pathways acting via P2Y₁₂ ADP receptor and cyclo-oxygenase.

This work was supported by the German Foundation for Heart Research (Deutsche Stiftung für Herzforschung, Frankfurt, Germany). Anneke Bierend was supported by a grant from the German Foundation for Heart Research. We are grateful to the Cardiovascular Working Group HerzInForm (Arbeitsgemeinschaft Herz-Kreislauf, Hamburg, Germany) for its help in recruiting patients. We thank Simone Bremer for competent secretarial help.

REFERENCES

- 1 Hankey GJ, Eikelboom JW. Aspirin resistance. *Lancet* 2006; 367: 606–17.
- 2 Wang TH, Bhatt DL, Topol EJ. Aspirin and clopidogrel resistance: an emerging clinical entity. *Eur Heart J* 2006; 27: 647–54.
- 3 Daniel JL, Dangelmaier C, Jin J, Ashby B, Smith JB, Kunapuli SP. Molecular basis for ADP-induced platelet activation. I. Evidence for three distinct ADP receptors on human platelets. *J Biol Chem* 1998; 273: 2024–9.
- 4 Hollopeter G, Jantzen HM, Vincent D, Li G, England L, Ramakrishnan V, Yang RB, Nurden P, Nurden A, Julius D, Conley PB. Identification of the platelet ADP receptor targeted by antithrombotic drugs. *Nature* 2001; 409: 202–7.
- 5 Kauffenstein G, Bergmeier W, Eckly A, Ohlmann P, Leon C, Cazenave JP, Nieswandt B, Gachet C. The P2Y₁₂ (12) receptor induces platelet aggregation through weak activation of the alpha (IIb) beta (3) integrin – a phosphoinositide 3-kinase-dependent mechanism. *FEBS Lett* 2001; 505: 281–90.
- 6 O'Donnell CJ, Larson MG, Feng D, Sutherland PA, Lindpaintner K, Myers RH, D'Agostino RA, Levy D, Tofler GH. Genetic and environmental contributions to platelet aggregation: the Framingham heart study. *Circulation* 2001; 103: 3051–6.
- 7 Cattaneo M, Zighetti ML, Lombardi R, Martinez C, Lecchi A, Conley PB, Ware J, Ruggeri ZM. Molecular bases of defective signal transduction in the platelet P2Y₁₂ receptor of a patient with congenital bleeding. *Proc Natl Acad Sci USA* 2003; 100: 1978–83.
- 8 Shiraga M, Miyata S, Kato H, Kashiwagi H, Honda S, Kurata Y, Tomiyama Y, Kanakura Y. Impaired platelet function in a patient with P2Y₁₂ deficiency caused by a mutation in the translation initiation codon. *J Thromb Haemost* 2005; 3: 2315–23.
- 9 Fontana P, Dupont A, Gandrille S, Bachelot-Loza C, Reny JL, Aiach M, Gaussem P. Adenosine diphosphate-induced platelet aggregation is associated with P2Y₁₂ gene sequence variations in healthy subjects. *Circulation* 2003; 108: 989–95.
- 10 Fontana P, Gaussem P, Aiach M, Fiessinger JN, Emmerich J, Reny JL. P2Y₁₂, H2 haplotype is associated with peripheral arterial disease: a case–control study. *Circulation* 2003; 108: 2971–3.
- 11 Ziegler S, Schillinger M, Funk M, Felber K, Exner M, Mlekusch W, Sabeti S, Amighi J, Minar E, Brunner M, Müller M, Mannhalter C. Association of a functional polymorphism in the clopidogrel target receptor gene, P2Y₁₂, and the risk for ischemic cerebrovascular events in patients with peripheral artery disease. *Stroke* 2005; 36: 1394–9.
- 12 National Center for Biotechnology Information. US National Library of Medicine. P2RY12 purinergic receptor P2Y, G-protein coupled, 12. Available at http://www.ncbi.nlm.nih.gov/entrez/query.fcgi?db=gene&cmd=Retrieve&dopt=full_report&list_uids=64805 (last accessed 16 November 2006).
- 13 Hetherington SL, Singh RK, Lodwick D, Thompson JR, Goodall AH, Samani NJ. Dimorphism in the P2Y₁ ADP receptor gene is associated with increased platelet activation response to ADP. *Arterioscler Thromb Vasc Biol* 2005; 25: 252–7.
- 14 Storey RF, Wilcox RG, Heptinstall S. Comparison of the pharmacodynamic effects of the platelet ADP receptor antagonists clopidogrel and AR-C69931MX in patients with ischaemic heart disease. *Platelets* 2002; 13: 407–13.

- 15** Aleil B, Ravanat C, Cazenave JP, Rochoux G, Heitz A, Gachet C. Flow cytometric analysis of intraplatelet VASP phosphorylation for the detection of clopidogrel resistance in patients with ischemic cardiovascular diseases. *J Thromb Haemost* 2005; 3: 85–92.
- 16** Maree AO, FitzGerald DJ. Variable platelet response to aspirin and clopidogrel in atherothrombotic disease. *Circulation* 2007; 115: 2196–207.
- 17** Born GVR, Cross MJ. The aggregation of blood platelets. *J Physiol Lond* 1963; 168: 178–95.
- 18** Angiolillo DJ, Fernandez-Ortiz A, Bernardo E, Ramirez C, Cavallari U, Trabetti E, Sabate M, Jimenez-Quevedo P, Hernandez R, Moreno R, Escaned J, Alfonso F, Banuelos C, Costa MA, Bass TA, Pignatti PF, Macaya C. Lack of association between the $P2Y_{12}$ receptor gene polymorphism and platelet response to clopidogrel in patients with coronary artery disease. *Thromb Res* 2005; 116: 491–7.
- 19** Paul BZ, Jin J, Kunapuli SP. Molecular mechanism of thromboxane A (2) -induced platelet aggregation. Essential role for $p2t$ (ac) and alpha (2a) receptors. *J Biol Chem* 1999; 274: 29108–14.
- 20** Jin J, Quinton TM, Zhang J, Rittenhouse SE, Kunapuli SP. Adenosine diphosphate (ADP) -induced thromboxane A (2) generation in human platelets requires co-ordinated signaling through integrin alpha (IIb) beta (3) and ADP receptors. *Blood* 2002; 99: 193–8.
- 21** Shankar H, Garcia A, Prabhakar J, Kim S, Kunapuli SP. $P2Y$ receptor-mediated potentiation of thrombin-induced thromboxane A generation in platelets occurs through regulation of $Erk1/2$ activation. *J Thromb Haemost* 2006; 4: 638–47.